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Exhibit I

Bradykinin antagonists: new opportunities

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The pro-inflammatory, pain producing, and cardiovascular effects of bradykinin B_2 receptor activation are well characterized. Bradykinin B_1 receptors also produce inflammation and pain. Therefore, antagonists are expected to b anti-inflammatory/analgesic drugs. Other exploitable clinical opportunities may exist. The newly discovered non-peptide B_2 receptor antagonists and the equivalent B_1 receptor pharmacological agents, which are in the pipeline, are suitable preclinical tools to properly evaluate potential utilities.

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Abbreviations
BK bradykinin
COX cyclo-oxygenase

Figure 1

1 H–D-Arg–Arg–Pro–Hyp–Gly–Igl–Ser–D-f5f–Igl–Arg–OH
2 H–D-Arg–Arg–Pro–Hyp–Gly–Thi–Ser–D-Tic–Oic–Arg–OH
3 Suc[Ser–D-Tic–Oic–Arg–OH]$_2$
4 H–D-Arg–D-Asp–[Ser–D-Tic–Oic–Arg–OH]$_2$
5 H–D-Arg–Arg–Pro–Hyp–Gly–Thi–c(Dab–D-Tic–Oic–Arg)

Current Opinion in Chemical Biology

B_2 peptide receptor antagonists (1–5; see text for full details). Dab, diaminobutryic; f5f, pentafluorophenylalanine; Hyp, 3-hydroxyproline; Igl, α-(2-indanyl)glycine; Oic, octahydroindole-2-carboxylic acid; Suc, succinyl; Thi, β-(2-thienyl)alanine; Tic, tetrahydroisoquinline-3-carboxylic acid.

Introduction

Bradykinin (BK) and related kinins are autacoid peptides produced by the catalytic action of kallikrein enzymes on plasma and tissue precursors termed kininogens. Kinins play integral roles in pathophysiological processes that accompany acute and chronic pain and inflammation. Their biological actions are mediated by at least two major G-protein-coupled BK receptors termed B_1 and B_2. The B_2 receptor is constitutively expressed on most cell types, whereas the B_1 receptors are not present in tissues under 'normal' conditions but are induced during inflammatory insults [1,2]. The putative role of kinins, and specifically BK, in the management of pain and inflammation has provided the impetus for developing potent and selective BK antagonists. In recent years, this effort has been heightened with the expectation that useful therapeutic agents endowed with analgesic and anti-inflammatory properties will be discovered.

In this review, which covers the literature from 1999 to March 2000, we discuss the chemical and pharmacological features of new B_1 and B_2 receptor antagonists, summarize advances in BK receptor physiology, and provide an outlook on the potential clinical opportunities of B_1 and B_2 antagonists.

B_2 peptide receptor antagonists

Antagonists for the BK B_2 receptor were discovered in the mid-1980s by incorporating point group substitutions at position 7 (Pro[7]) of the BK nonapeptide [3]. Further replacem nts of key amino acids resulted in additional potency gains; however, the resulting peptides suffered from lack of selectivity for the B_2 receptor, were rapidly degraded, and showed partial agonist activity [3]. To remedy these shortcomings, second- and third-generation peptide antagonists containing non-proteinogenic amino acids were introduced [4]. The unprecedented level of potency that these peptide antagonists display for the human kinin B_2 receptor has continued to fuel the search for analogues, especially for those with improved overall pharmacological profiles. A recent advance with regard to potency and stability is the discovery of B-10056 (1, Figure 1) [5]. This derivative contains a pentafluorophenylalanine residue at position 7 and is among the most potent antagonists reported with pA_2 values (the negative logarithm of the molar concentration of antagonist that causes doubling of the EC_{50}, compared with the EC_{50} in the absence of antagonist) in rat uterus and guinea pig ileum of 10.5 and 8.0, respectively. However, 1 shows combined B_1/B_2 receptor antagonist activity.

The 'bivalent ligand' approach, which has been shown to increase the potency and duration of action of bioactive compounds, has also been applied to the design of new BK B_2 receptor antagonists [6]. Beginning with HOE 140 (Icatibant, 2, Figure 1) as the parent structure, various carboxy-terminal fragments were dimerized utilizing an amino-terminal succinyl linker. In this way, the carboxy-terminal tetrapeptide of 2 was transformed to 3 (Figure 1), which has low but significant affinity for the human B_2 receptor (K_i 6.6 μM); by contrast, the monomeric counterpart, H–Ser–D-Tic–Oic–Arg–OH presents no detectable affinity up to a 10 μM concentration. Further linker modifications led to analogue 4 (Figure 1). This compound competitively inhibits the binding of BK to cloned human B_2 receptors with K_i = 76 nM. None of the reported dim rs have significant affinity for human cloned B_1 receptors; yet, it appears unlikely that a breakthrough compound will em rge from this compound [illegible]

Because of its extraordinary B_2 receptor affinity, 2 has served as a point of reference for almost a decade and continues to provide the inspiration for the design of new B_2 antagonists. MEN 11270 (5, Figure 1) is a recently reported derivative of 2 that binds to the B_2 receptor constitutively expressed by WI38 human fibroblasts with a pK_i value of 10.3 [7]. Compound 5 is related to 2 in that the carboxy-terminal region has been cyclized to constrain it to a β-turn conformation. All indications are that 5 has a pharmacological profile comparable to that of 2, supporting the idea that a β-turn arrangement at the carboxy-terminus preserves the ligand–B_2-receptor interaction. Knowing and understanding the bioactive conformation of 2 might be a prelude to the rational design of novel nonpeptide B_2 antagonists.

B_2 nonpeptide receptor antagonists

A thorough assessment of the role of kinins in human pathologies is more likely to be made with low molecular weight, orally bioavailable agents that are protected from rapid metabolic degradation than with peptide antagonists whose potential for therapeutic use is ultimately limited. These factors have provided the impetus for developing non-peptide B_2 receptor antagonists. By using a directed random screen of angiotensin II AT_1 receptor antagonists, and by exploiting the relationship between angiotensin II and BK, researchers from Fujisawa Pharmaceutical (Ibaraki, Japan) identified a number of quinoline and imidazo[1,2-a]pyridine derivatives that possess high binding affinity for kinin B_2 receptors [8••,9]. Refinement of these lead structures and extensive structure/activity relationship investigations led to the recently characterized antagonist Fr 172357 (6, Figure 2) [10]. This compound acts as a competitive antagonist in human umbilical vein ($pA_2 = 8.65$), in rat jugular vein ($pA_2 = 9.07$), and as a non-competitive antagonist in pig coronary artery ($pK_B = 10.14$). Relative to other B_2 receptor antagonists, 6 is very potent; unfortunately, it exhibits some cross-reactivity as it also interacts with the tachykinin NK_1 receptor in rabbit and porcine vasculature.

Building on the B_2 antagonist structures delineated by the researches at Fujisawa, the Hoechst Marion Roussel group (Frankfurt, Germany) reported the discovery of two new series of compounds, *ortho*-substituted 8-quinoline and 4-benzothiazole derivatives [11]. The affinities for the B_2 receptor were marginally higher for the quinolines relative to the corresponding benzothiazoles, with the most potent analogue, 7, exhibiting a K_i value of 0.1 nM in a guinea pig membrane preparation.

Other recently introduced compounds related to 6 and 7 derive from the Fournier (Dijon, France) effort in this area. LF 16-0687 and LF 16-0335C (8 and 9, respectively, Figure 2) are both potent ($K_i = 0.67$ nM and 0.84 nM, respectively; human B_2 receptor expressed in CHO cells), selective, and competitive antagonists at the kinin B_2 receptor [12,13]. Their distinguishing structural features include the presence of basic phenylamidine moieties on one terminus and a central sulfonamide linkage replacing the *N*-methyl amide group present in 6 and 7. Compound 9 binds equally to the human, rat, and guinea pig B_2 rec ptor but displays different *in vivo* potencies in the latter two species.

The β-turn structural motif that has been postulated for the biologically active conformation of 2 formed the basis of a *de novo* design approach that led to non-peptide B_2 antagonists containing a 1,4-benzodiazepin-2-one scaffold [14]. The optimum compound is the racemic benzodiazepine 10 (Figure 2), wherein the guanidine residue and lipophilic benzodiazepine core resemble the positively charged Arg^9 residue and the hydrophobic residues D-Tic^7 and Oic^8 in 2. Further structural refinements are await d, as 10 exhibits only a moderate K_i value of 8.9 μM at the human B_2 receptor.

The structurally distinct B_2 antagonist bradyzid (11, Figure 2) evolved from a high-throughput scre ning lead and may be envisioned as a hybrid between the pr - lylamide section of the Fournier analogue 8 and the lipophilic benzodiazepine core of 10 [15]. The compound is orally bioavailable and selective for B_2 over B_1 receptors; however, it is much less potent at the human than at the rodent B_2 receptor ($K_i = 772$ nM compared with 0.89 nM, respectively; COS cells) [16]. As such, 11 will be a useful pharmacological tool but an unlikely candidate for development as a therapeutic agent.

The structural diversity among non-peptide kinin B_2 antagonists was extended with the synthesis of piperazine compounds derived from the anti-histamine drug, cetirizine [17]. Among the most potent analogues synthesized was 12 (Figure 2), which showed inhibition of BK (45.6% at 100 nM); however, 12 and its homologues all demonstrated some anti-histamine effects.

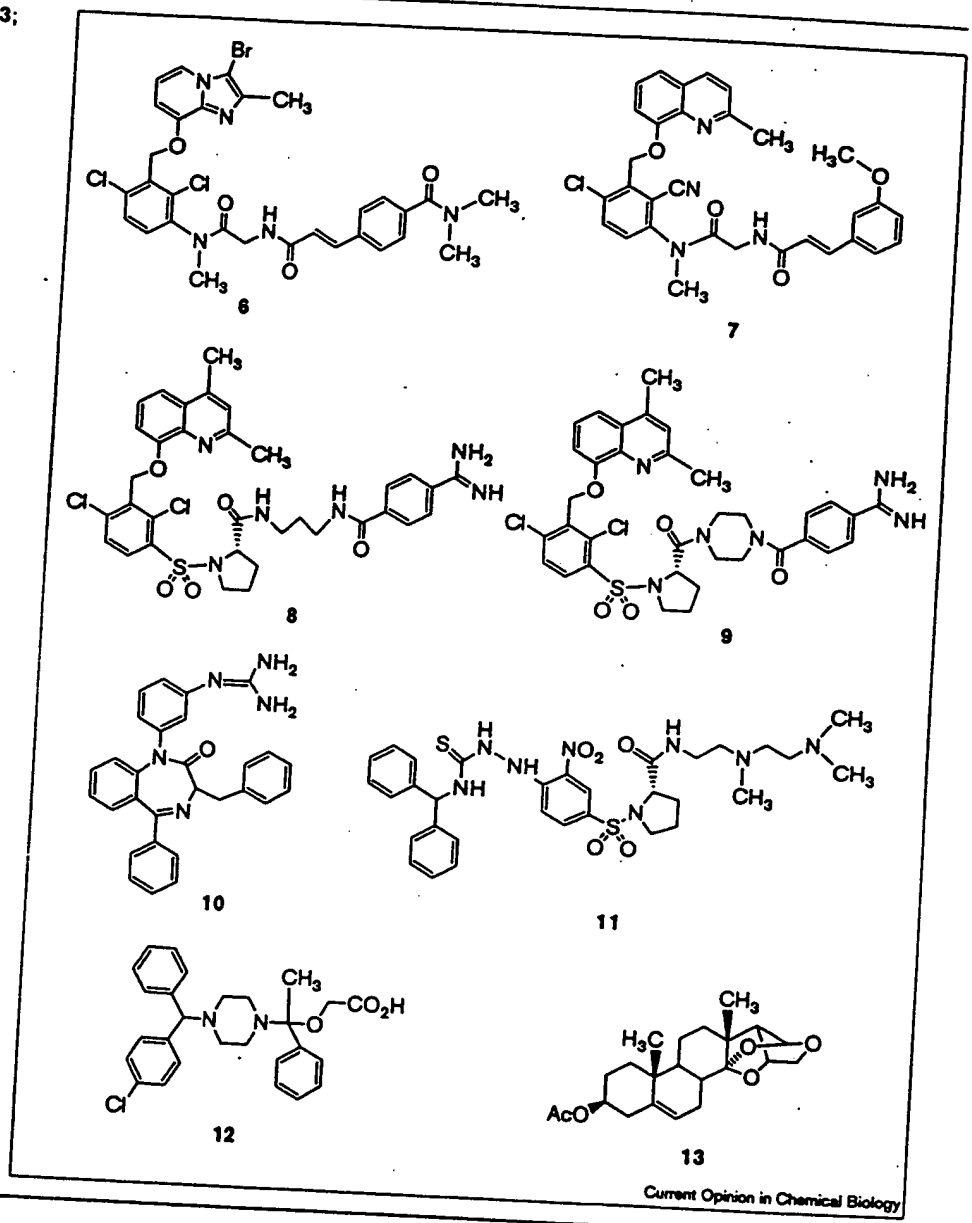
The isolation and characterization of a novel non-peptide B_2 antagonist from natural sources was reported recently [18]. Acetylillustrol, (13, Figure 2) when injected into rat paw (10 nmol/paw), causing a significant inhibition of carageenan and BK-induced rat paw edema. At the same dose, 13 failed to alleviate the edema caused by the B_1-selective kinin agonist, des-Arg^9-BK.

B_1 peptide receptor antagonists

Peptide antagonists for the kinin B_1 receptor were discovered almost a decade before their B_2 receptor counterparts [19]. It was possible to increase the potency and stability of these first-generation compounds with specific amino acid substitutions (e.g. des-Arg^{10} HOE 140, 14, Figure 3) and these observations have sustained the search for further improvements. Recent efforts have focused on identifying critical residues in 14 by performing an alanine scan [20]. This work has singled out position 3 (Pro) and the carboxy-terminal dipeptide (D-Tic–Oic) as the most sensitive with regard to activity. Further potency and selectivity gains for 14 have been realized by incorporating a linear, flexible

Figure 2

B_2 non-peptide receptor antagonists (6–13; see text for full details).



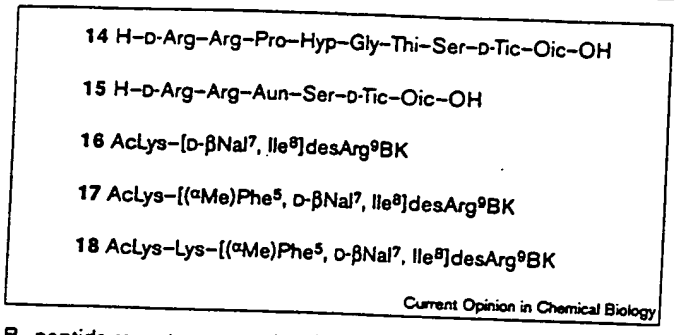
alkyl linker to connect two terminal peptide fragments [21]. An analog of 14, MEN 11575 (15, Figure 3), containing an 11-aminoundecanoic acid spacer, is more potent as a kinin B_1 antagonist in a rat ileum longitudinal smooth-muscle assay than its unmodified precursor 14 (pA_2 7.1 versus 6.8). Additionally, 15 is devoid of ancillary activity displayed by the parent peptide.

To lessen the susceptibility of th previously report d kinin B_1 antagonist R715 (16, Figure 3) to proteolytic degradation, derivatives containing α-methyl-L-phenylalanine were prepared [22]. Two congeners of 16 (17 and 18, Figure 3) show high antagonistic potencies at the human B_1 receptor (pA_2 = 8.8 and 8.7, respectively) and are resistant to *in vitro* degradation by purified angiotensin-converting enzyme from rabbit lung and amino peptidases from human plasma.

B_1 non-peptide receptor antagonists

There are indications that the development of non-peptide kinin B_1 antagonists is beginning to receive attention, although progress in this area has been slow. Since a 1997 patent claiming non-peptide B_1 antagonists was issued by Sanofi [P1*], only two additional accounts have been published. Fournier revealed that an early-stage effort to develop orally active non-peptide B_1 antagonists was underway with potential in asthma; no additional details were provid d [23]. Similarly, scientists at Pharmacopeia (Princeton, NJ) r port d the discovery, by screening encoded combinatorial libraries, of a potent non-peptide kinin B_1 antagonist [24]. The compound, PS020990, is allegedly >1000-fold selective for the kinin B_1 and a variety of other receptors; no structure has been disclosed.

Figure 3



B_1 peptide receptor antagonists (14–18; see text for full details). AcLys, *N*-acetyl lysine; Aun, aminoundecanoic acid; β-Nal, 3-(2-naphthyl)alanine.

Potential clinical utilities of BK receptor antagonists

It is generally believed that B_2 receptors, but not B_1 receptors, are expressed in normal tissues and that inflammation, tissue damage or bacterial infection can rapidly induce B_1 receptor expression. This makes the B_1 receptor a particularly attractive drug target. B_1 receptor signaling may have different, more persistent consequences than B_2 receptor signaling. Studies with cell lines expressing recombinant receptors showed that B_2 receptors are more prone to desensitization, showing rapid (i.e. within minutes) ligand internalization, loss of cell surface receptors and consequently loss of functional responsiveness, and long term receptor down-regulation [25]. This property is likely to occur in native tissues because it resides in the amino acid code in the intra-cellular tail of the receptor [25]. For instance, in cultured rat dorsal root ganglion neurons, ionic currents evoked by BK are short lived and fade even during the period of agonist application [26]. In contrast, recombinant B_1 receptors show no ligand internalization or receptor sequestration leading to persistent activation and long term up-regulation [25].

At first sight, there are some concerns about the utility of the B_2 receptor as a drug target because this receptor seems to be involved in normal cardiovascular regulation and this issue has become prominent with the use of B_2 receptor knockout mice. These animals show elevated heart rate and ultimately develop cardiomyopathy, effects which have an earlier onset in homozygote (–/–) compared to heterozygous (+/–) knockout animals showing a gene dose effect [27•]. The common criticism that this is merely a consequence of the genetic manipulation is outweighed by observations that, in wild-type mice, administration of icatibant (2, Figure 1) increased blood pressure to the same level seen in the homozygote knockout animals [27•]. These data strongly suggest that, in mice at least, B_2 receptors regulate normal cardiovascular function and preserve cardiac structure [28]. It is these aspects of B_2 receptor pharmacology that are worrisome. On the other hand, in animals, BK has deleterious effects in coronary ischaemia. In transgenic mice harboring the human B_2 receptor gene, depressor responses to BK injection are enhanced compared with in wild-type litt r-mates [27•]. Furthermore, a recent report suggests that the cardioprotective effects of angiotensin converting enzyme (ACE) inhibitors may not be mediated by elevation of BK levels, as was first thought [29]. The controversy is reviewed by Dell'Italia and Oparil [30•].

In favor of the development of B_2 receptor antagonists is the recent observation that B_2 receptor expression is 'inducible'. For instance, reverse transcriptase PCR studies failed to detect B_2 receptor mRNA in dorsal root sensory ganglion neurons either freshly disassociated or cultured in the absence of nerve growth factor; yet, strong expression was detectable in the presence of nerve growth factor [31]. It is unknown whether other inflammatory mediators can up-regulate B_2 receptor expression, and this may have important consequences on the long-term sensitization of nociceptors in inflammatory conditions (see below). In addition, in cultured human bronchial smooth muscle cells, B_2 receptor expression was induced by interleukin-1β [32]. B_2 receptor activation may also 'prime' B_1 receptor induction [33]. For example, in rats, the edema response produced by intradermal injection of BK was l st on repeated injection; however, the same injection site developed responsiveness to the B_1 receptor agonist desArg^9 BK (see [34]).

The role of BK in pain is beyond doubt. BK, acting as an agonist at B_2 receptors, is one of the most potent algogenic (i.e. pain) producing substances. Not only does it excite nociceptors directly, but it also sensitizes them to other stimuli including inflammatory mediators and heat [26]. Sensitization is mediated by a different mechanism to the way that BK produces direct excitation and, unlike the excitatory effects, nociceptor sensitization does not show rapid desensitization [26]. Thus, B_2 receptor antagonists are expected to be analgesic. The pro-inflammatory action of B_2 receptors, namely increased microvascular leakage, has been exploited using a peptide dimer BK agonist to produce modest increases in the permeability of the blood–brain barrier, thereby improving access of chemotherapeutic agents to brain tumors that would normally be protected (see [35]). Overall, given the recent findings about B_2 receptor desensitization and the inducible nature in inflammation, it would be of interest to re-assess the potential clinical utilities and side-effect profile of the newer, orally active, high affinity B_2 receptor antagonists.

In animals, B_1 receptor agonists produce hyperalgesia, an effect blocked by peptide B_1 receptor antagonists (for review see [36]). However, there is little evidence for excitation or sensitization of nociceptors by B_1 receptor agonists. Eckert *et al.* [37] reported that nerve ligation up-regulates B_1 receptor binding sites in dorsal root ganglia; however, their function is unknown. It has be n postulated that the pain-producing effects of B_1 receptors are mediated through inflammatory cells releasing substances

that then sensitize nociceptors. The ability of B_1 receptor antagonists to block the cellular inflammatory response may be an important and clinically exploitable anti-inflammatory mechanism [34]. In multiple sclerosis, the migration of inflammatory cells into the central nervous system is a stimulus for lesion development and a process dependent on the endothelial cell/lymphocyte interaction [38] initiates this. *In vitro* studies have shown that B_1 receptors are up-regulated in T-lymphocytes obtained from multiple sclerosis patients and that these receptors alter T-cell migration [35]. *In vitro*, des.Arg^{9}Leu^8-BK, but not 2, prevented neutrophil migration initiated by cytokines (see [34]). Further experiments with non-peptide antagonists are required to tease out whether B_1 r ceptor antagonists are purely anti-inflammatory or whether they also possess analgesic properties, and then predictions on their clinical utility can be made.

The potential of B_1 and/or B_2 receptor antagonists as anti-inflammatory, analgesic drugs is exciting because there is great clinical need for new drugs of this class. The recent introduction of selective cyclo-oxygenase (COX)-II inhibitors led to expectations of an improved side-effect profile compared with that associated with the prolonged use of non-steroidal anti-inflammatory drugs. In this context, COX products such as prostaglandin E2 induce B_1 receptor expression, and B_1 receptor activation (by stimulation of COX) can itself release prostanoids. It is not known whether B_1 receptors operate independently of COX and whether B_1 receptor antagonists would have a different pharmacological, anti-inflammatory, analgesic profile [39], although B_1 receptor antagonists may offer advantages in terms of a lowered side-effect profile. The potential for renal side-effects of selective COX-II inhibitors remains because of the constitutive expression of this enzyme in kidney. In rat kidney, although B_1 receptor mRNA can be induced by pre-treatment with lipopolysaccharide, B_1 receptors are not present under normal conditions [33]. Interestingly, considering the areas of pharmacological overlap, non-steroidal anti-inflammatory drugs have been used to inhibit the growth of some cancers and it is possible that B_1 and/or B_2 receptor antagonists may share this property. Indeed BK antagonists can induce apoptosis in cancerous cells [4]. The clinical utility of this 'anti-cancer' action warrants further examination.

Conclusions

There are recognizable differences in the roles of B_1 and B_2 receptors and the clinical exploitation of each subtype should be explored with the development of both B_1 or B_2 selective receptor antagonists. Of particular importance is the fact that B_2 receptors directly excite and sensitize nociceptors and therefore, B_2 receptor antagonists are expected to be analgesic. However, concerns about the cardiovascular liabilities of B_2 receptor antagonists should be properly evaluated. There are also considerable areas of overlap in B_1 and B_2 receptor pharmacology, particularly in their pro-inflammatory actions. The constitutive expression (but rapid desensitization) of B_2 receptors versus the inducible nature of B_1 rec ptors (coupled with persistent signaling) implies these receptors have different roles in the course of an inflammatory process. Therefore, a mixed B_1/B_2 receptor antagonist might be a suitable drug development target. Such a dual antagonist approach was shown to be a more successful strategy in an animal model of sepsis [4]. Although the dual antagonist Bradycor did not achieve sufficient clinical efficacy in patient trials, this may have been due to the metabolic liability and low potency of this peptide dimer [4]. Repetition of these experiments with a more suitable drug candidate would be scientifically meritorious.

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Patent

P1. Ferrari B, Gougat J, Muneaux C, Muneaux Y, Perreaut P,
• Planchenault C: **Nouveaux derives de *N*-(arylsulfonyl)aminoacides ayant une affinite pour les recepteurs de la bradykinine.** 1997, WO 97/25315. [Title translation: New derivatives of *N*-(arylsulfonyl) amino acids with an affinity for bradykinin receptors.]
This patent discloses the first non-peptide B_1 antagonists.